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Pectin/poly(lactide-co-glycolide) composite matrices for biomedical applications

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Abstract

The aim of the research was to develop matrices for the delivery of biologically active substances for tissue regeneration. To this end, a new biodegradable matrix composed of a hydrophobic porous poly(lactide-co-glycolide), p(LGA), network entangled with another network of hydrophilic pectin was fabricated in the presence of calcium chloride. The calcium salts function as both a pore forming reagent and cross-linker for the formation of pectin networks; the method combines creating pores and cross-linking polymers in one step. Microscopic imaging and dynamic mechanical analysis revealed a double-network structure of the composite matrices. The pectin enables the composite to carry signal molecules. This is accomplished by linking signal molecules to pectin by physical adsorption or by chemical reaction. The p(LGA) networks in the composite impart mechanical properties comparable to p(LGA) alone. The mechanical properties of the composite are far superior to matrices containing only pectin. Furthermore, the pectin-containing matrices improved cell adhesion and proliferation when compared to plain p(LGA) matrices, as determined in vitro by osteoblast culture.
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Keywords: Pectin; Poly(lactide-co-glycolide); Matrix; Biomaterials; Tissue engineering

1. Introduction

Pectins are polysaccharides enriched in galacturonic acid and galacturonic acid methyl ester units. Combined with proteins and other polysaccharides, pectins form skeletal tissues of plants, which are chemically stable and physically strong [1,2]. With high molecular weight and a polyanionic nature, pectins react to their environments through a continuum of physical states, ranging from dense gels to dilute solutions. These properties enable pectin polymers to carry signal molecules and support various biologically active substances. In addition, pectins closely imitate the structure of polysaccharides found in the extracellular matrices of mammals. Thus, their properties and general availability make pectins viable to consider when engineering new biomedical materials.

Pectins have shown promise in engineering drug carriers for oral drug delivery [3]. The combination of pectin and a second polymer into a composite may alter degree of swelling [4,5], and change mechanical properties [6]. New uses of pectins in biomedical applications include facilitating the delivery of specific sequences of amino acids, anti-inflammatory agents, anti-coagulants, and wound healing substances to tissue sites. To be used as such, pectin based composites can be formed into membranes, microspheres, scaffolds, or injectable gels. Here, we describe the development and characterization of composite matrices of pectin and poly(lactide-co-glycolide), pectin/p(LGA).

P(LGA) has been used clinically for tissue repair and organ regeneration for decades. This hydrophobic polymer is biocompatible, biodegradable, and easily processed into a variety of sizes and shapes which have good mechanical properties [7–10]. Although p(LGA) will support cell attachment and cell growth, it does not impart signals to the cells [11]. The inability to convey signals limits the application of p(LGA). This deficiency is currently overcome by synthesizing block or graft copolymers of lactic acid and lysine or other segments carrying side chain functional groups [11,12]. Through

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the functional groups, specific amino acid sequences can be attached. By this strategy, a number of new chemical entities have been provided. The preparation of copolymers involves a series of isolation, purification and identification procedures.

In this study, we present a method to prepare porous matrices from pectins and p(LGA) that retain the biomechanical strength of p(LGA) yet provide access for hydrophilic, bioactive substances. The matrices were characterized for structural, physical and mechanical properties, and the capacity to store signal molecules. Furthermore, these matrices were tested in vitro for the ability to support cell attachment and growth. These properties are required in tissue engineering applications.

2. Experimental

2.1. Materials

Sodium salts of pectin from citrus fruits (degree of esterification, DE, 93%), bovine serum albumin (BSA), fluoresceinamine, p(LGA) [50:50; average M_w, 50,000–75,000; T_g 45–50°C], pectinase 3XL, neutral-buffered formalin, trypan blue, DNA Quantitation Kit, 2,2,2,-trifluoroethanesulfonyl chloride (tresyl chloride), and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum, α-minimum essential medium (α-MEM), ascorbic acid-free α-MEM (Formula 94-5049EL), penicillin–streptomycin, Dulbecco's phosphate-buffered saline, and trypsin-EDTA were purchased from Gibco BRL Products, Life Technologies (Grand Island, NY). Ascorbic acid was purchased from Fisher Scientific (Pittsburgh, PA). Micro BCA* reagent was from Pierce (Rockford, IL). Ethylene oxide was purchased from H.W. Anderson Products (Chapel Hill, NC). All other chemicals were A.C.S. grade, and used without further purification.

2.2. De-esterification of pectins

Gentle alkaline de-esterification was performed by adjusting the pH of a pectin solution (1%, w/v) to 8.0 with 0.1 N NaOH and stirring at 4°C over 48 h [13]. The reaction solution was dialyzed against a large volume of distilled water (DI water). Pectins were recovered by spreading the pectin solution into ethanol containing 0.1% $CaCl_2$, the resultant microparticles were filtered, washed with DI water, and lyophilized. Pectin particles with the size ranging from 15 to 125 μm were collected. The extent of de-esterification was determined by comparing the DE values of the de-esterified pectins with those before the reaction. The DE values of pectins were measured by high-performance liquid chromatography (HPLC) as previously reported [14]. Other molecular properties of the de-esterified pectins, such as the weight average M_w, root mean square radius of gyration (R_{gz}), and intrinsic viscosity ([η]), were evaluated by HPSEC with on-line multi-angle laser light scattering and viscometric detection as described previously [15].

2.3. Preparation of pectin/p(LGA) composites

Pectin/p(LGA) composite matrices were prepared by a multi-step procedure. In step I, 1.0 g of p(LGA) was dissolved in 8.0 ml of chloroform, into which 0.10 g of de-esterified pectin, 2.0 g of calcium chloride, and 6.9 g of sodium chloride were dispersed and blended to form a slurry. The size of the inorganic salt particles ranged from 50 to 200 μm. In step II, the slurry was cast into disks in a mold with dimensions of 6 mm in diameter and 3 mm in thickness, and the solvent was evaporated to form a solid matrix. In step III, the matrix was immersed in 1 l of deionized water (DI water), where pectin particles started to swell and hydrate, salts began to dissolve and diffuse. Meanwhile, dissolved calcium ions reacted with and bound to the hydrated pectin particles via inter- and intra-chain chelation. Dissolved sodium chloride and excessive calcium salts diffused to create spaces for water migration. The process in step III was continued for 48 h. In that time the water was changed several times to complete cross-linking of pectin and leaching of residual salts. Lastly, freeze drying the matrices created a channeled porous structure.

Porous p(LGA) matrices were prepared by the same method as described above, except for the substitution of pectin with sodium chloride. Porous pectin matrices were prepared by casting pectin solution (2.0%, w/v) in a mold (6×3 mm ($d \times h$)) lyophilizing the solution to create a solid structure, which thereafter was treated with calcium chloride solution (0.1 M) and lyophilized. The p(LGA) and pectin matrices were used as controls.

2.4. Recovery of p(LGA) and pectin from pectin/p(LGA) matrices

Samples were analyzed to determine the efficiency with which calcium chloride cross-linked pectin particles and the amounts of pectin and p(LGA) in the final composite matrices. Samples of pectin/p(LGA) matrices were vacuum-dried for 24 h prior to experimentation. Each dried sample was placed in 2.0 ml tetrahydrofuran (THF) in a volumetric flask equipped with a pennyhead stopper to prevent solvent evaporation. The mixture was gently shaken at room temperature for 2 h to complete the extraction of p(LGA) polymers. The extraction solution was removed and analyzed for p(LGA) content using a Shimadzu HPLC equipped with an RID-10A refractive index detector and an SCL-10A data station

(Model LC-10AD, Kyoto, Japan). An aliquot of the solution (10 μl) was injected and eluted by THF on a phenogel guard column (model 22824G, 50 × 7.8 mm, Phenomenex, Torrance, CA) and a phenogel column (model GP/4446, 300 × 7.8 mm, Phenomenex) at the flow rate of 0.5 ml/min. p(LGA)/THF solutions of known concentrations were run under the same conditions and used to prepare a standard curve.

After the removal of p(LGA) polymers, the solid residues, calcium cross-linked pectins, were washed with fresh THF (2 × 2 ml), dry ethanol (3 × 2 ml), and air-dried. Sodium phosphate solution (1.0 M, 2.0 ml, pH 6.5) was added to the flask and sonicated to solubilize the pectin. Pectin content was analyzed by total sugar assay [16,17].

2.5. Chemical modification of pectin/p(LGA) matrices

The chemical modification of pectin/p(LGA) composite matrices was performed by grafting the matrices with fluoresceinamine using tresyl chloride as a coupling reagent as previously reported [18,19]. Samples of pectin/p(LGA) matrix were immersed in dry acetone (pre-dried over molecular sieve 4A; Acros, Pittsburgh, PA) for 24 h with three changes. To a glass vial containing 2.0 ml dry acetone and one piece of the dry sample, pyridine (200 μl) and tresyl chloride (100 μl) were added, and gently shaken for 10 min at room temperature. The sample was removed and rinsed with dry acetone (3 × 5 ml), phosphate-buffered saline (PBS) (pH 7.0, 2 × 5 ml), and placed in 2.0 ml of PBS (pH 7.8) containing fluoresceinamine (20 mM) and incubated for 20 h at room temperature. To completely remove the fluoreceinamine which was physically adsorbed rather than chemically conjugated, the sample was washed with 1 mM HCl and 0.2 M $NaHCO_3$ repeatedly, and 1.0 M NaCl containing 1 mM HCl, finally with DI water [19]. Samples thus treated were examined by confocal laser fluorescence microscopy as described in the following section.

P(LGA) matrices treated with both tresyl chloride and fluorescienamine under the same conditions were used as controls.

2.6. Microscopic imaging

2.6.1. Scanning electron microscopy (SEM)

For SEM examinations, samples of pectin particles, NaCl–$CaCl_2$ crystal mixtures, pectin/p(LGA), and p(LGA) matrices were mounted on specimen stubs, coated with a thin layer of gold in a sputter coating apparatus (Edwards High Vacuum, Wilmington, MA), and examined in a model JSM 840A scanning electron microscope (Jeol USA, Peabody, MA) operating at 10 kV in the secondary electron imaging mode. Images were collected at 25 × and 250 × using an Imix-1 digital image workstation (Princeton Gamma-tech, Princeton, NJ).

2.6.2. Confocal laser microscopy

Samples of fluorescently labeled pectin/p(LGA) composite matrices were glued to 1 × 3 inch microscope slides and placed in the sample stage of an IRBE optical microscope with a 10 × lens integrated with a model TCS-SP laser scanning confocal microscope (Leica Microsystems, Exton, PA). The parameters for the image acquisition were set for confocal reflection (633 nm) and confocal fluorescence (488/500–530 nm) in two channels.

2.7. Dynamic mechanical analysis (DMA)

Compressive mechanical testing of the matrices was performed on a Rheometric Scientific RSA II Solids Analyzer (Rheometric Scientific, Piscataway, NJ) fitted with 25 mm parallel plates. Temperature control was maintained using a liquid nitrogen environmental controller. Each sample matrix was placed on the lower plate, the upper plate was lowered onto the sample to give a slight compressive force, and then locked in the place. The samples were tested using a compressive strain of 0.25–1.0%, depending on the stiffness of the sample. Storage modulus, loss modulus, and loss tangent were determined over a temperature range of $-100^{\circ}C$ to $+200^{\circ}C$ at the heating rate of $10^{\circ}C$/min. The data were analyzed using Rheometric Scientific Orchestrator software, version 6.5.7.

2.8. Determination of equilibrium water content and protein adsorption

Samples of pectin/p(LGA) and p(LGA) matrices were dried under vacuum at room temperature for 24 h. prior to the experiment. Each dried sample was incubated with a large volume of PBS (pH 7.0) at room temperature under gentle shaking. Samples were removed from the incubation solutions at intervals of 5, 15, 30, 45 min, and 1, 2, 4, 8 h., rinsed three times with DI water, wiped with tissue paper to remove the water adsorbed on the surfaces, and weighed (W_w). The samples were then re-dried under vacuum for 24 h. and weighed (W_{rd}). The water content of matrices at each time point was calculated: water content = $(W_w - W_{rd})/W_w \times 100\%$.

The kinetics of protein adsorption in pectin/p(LGA) and p(LGA) matrices was studied by a procedure similar to that used for the determination of equilibrium water content, except for the addition of BSA (0.1%, w/v) in PBS. After rinsing with DI water, the samples were analyzed for the amount of protein adsorbed by protein BCA assay [20]. A series of BSA solutions with known concentrations were used to prepare a standard curve.

2.9. In vitro cell culture and bioassays

The potential for application of composite matrices in tissue engineering was evaluated in vitro by seeding and culturing osteoblast cells on the matrices. Osteoblasts (MC3T3-E1, clone 26) were thawed, cultured in a supplemented ascorbic acid-free α-MEM and 10% fetal calf serum (FBS) containing 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C with 5% CO_2. The medium was changed every other day. The cells of passages 3 and 4 were harvested, pelleted by centrifugation and re-suspended at the concentration of 2×10^6 cell/ml in α-MEM containing FBS (10%), antibiotics (1%), and L-ascorbic acid (50 mg/l) (complete medium). The viability of the cells was higher than 90% as determined with the trypan blue exclusion assay.

The pectin/p(LGA) and p(LGA) matrices were sliced into disks with dimensions of 6 mm in diameter and 1.5 mm in thickness, and sterilized in culture flasks with ethylene oxide for 2 days. The matrices were soaked in ethanol for 30 min, exchanged with PBS for three times for 30 min each time, then washed with the complete medium twice for 2 h each time.

For the cell attachment test, each of the matrices were placed in a teflon plate containing 0.5 ml of the cell suspension, cultured on an orbital shaker (Model 3520; Lab-Line Instrument, Melrose Park, IL) at 75 rpm under standard conditions. At day 3, the cell-loaded matrices were transferred into six-well tissue culture plates, 4 ml of complete medium were added into each well, cultured under standard conditions for 1 day. The matrices were removed from the medium, washed with PBS, fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin using standard procedures. Paraffin-embedded specimens were sectioned into 5-μm thick through the center, stained with hematoxylin and eosin, and examined under a light microscope [8,21].

For cell proliferation studies, the cell culture was continued for additional 7 and 14 days. The medium was changed every other day. At the conclusion of cell culture, the matrices were removed, washed with PBS, homogenized using a polytron homogenizer (Brinkmann Easycare Generator; Polytron-Aggregate, Switzerland) for 30 s at top speed (VI) for three times, then subjected to DNA assay for cell number quantitation [8,9]. DNA assays were performed using DNA Quantitation Kit with Hoechst 33258 dye. The concentration of DNA in solution was converted to a cell number using a conversion factor of 7.8 pg of DNA per MC3T3-E1 cell. This conversion factor was determined by measuring the amount of DNA from a known cell number.

2.10. Statistical analysis

The data presented here are mean ± standard deviation. To test the significance of observed differences between the study groups, a paired Student's *t*-test was applied.

3. Results and discussion

3.1. De-esterification of pectin

Pectins were de-esterified prior to use for preparation of pectin/p(LGA) composite matrices. An almost complete de-esterification was accomplished (Table 1) to enable the insolubilization of pectin macromolecules by calcium ions [22]. The M_w and intrinsic viscosity of the pectin were slightly reduced after de-esterification in comparison with the original polymer (Table 1). In addition, R_{gz} of pectin was reduced slightly after its de-esterification (Table 1). This indicated that some disaggregation and/or degradation of pectin macromolecules occurred during its de-esterification. Nevertheless, this seems to not significantly effect the binding efficiency of pectin to calcium ions, since more than 80% of the pectin suspended in the p(LGA)/chloroform solution was recovered from the resulting pectin/p(LGA) matrices. Also the ratio of pectin to p(LGA) polymers was reduced only slightly after matrix fabrication (Table 2).

Table 1
Molecular properties of pectins

Properties	De-esterification	
	Before	After
$M_w \times 10^{-5}$	0.87 (0.02)	0.81 (0.01)
R_{gz} (nm)	24.7 (2.06)	21.4 (0.06)
$[\eta]_w$ (dl/g)	1.25 (0.02)	1.22 (0.02)
DE (%)	93	10.2 (0.77)

Table 2
Physical characterization of pectin/p(LGA) and p(LGA) matrices

Matrices	Density (g/ml)	Pectin content (mg)		P(LGA) content (mg)	
		Calculated	Determined	Calculated	Determined
Pectin/p(LGA)	0.190	1.28	1.06 ± 0.2	12.8	11.4 ± 3.2
P(LGA)	0.306	N/A	N/A	26.0	24.8 ± 2.4

De-esterified pectins in the form of microparticles were collected and used for pectin/p(LGA) matrix preparation. The pectin particles showed some variations in size and morphology (Fig. 1A).

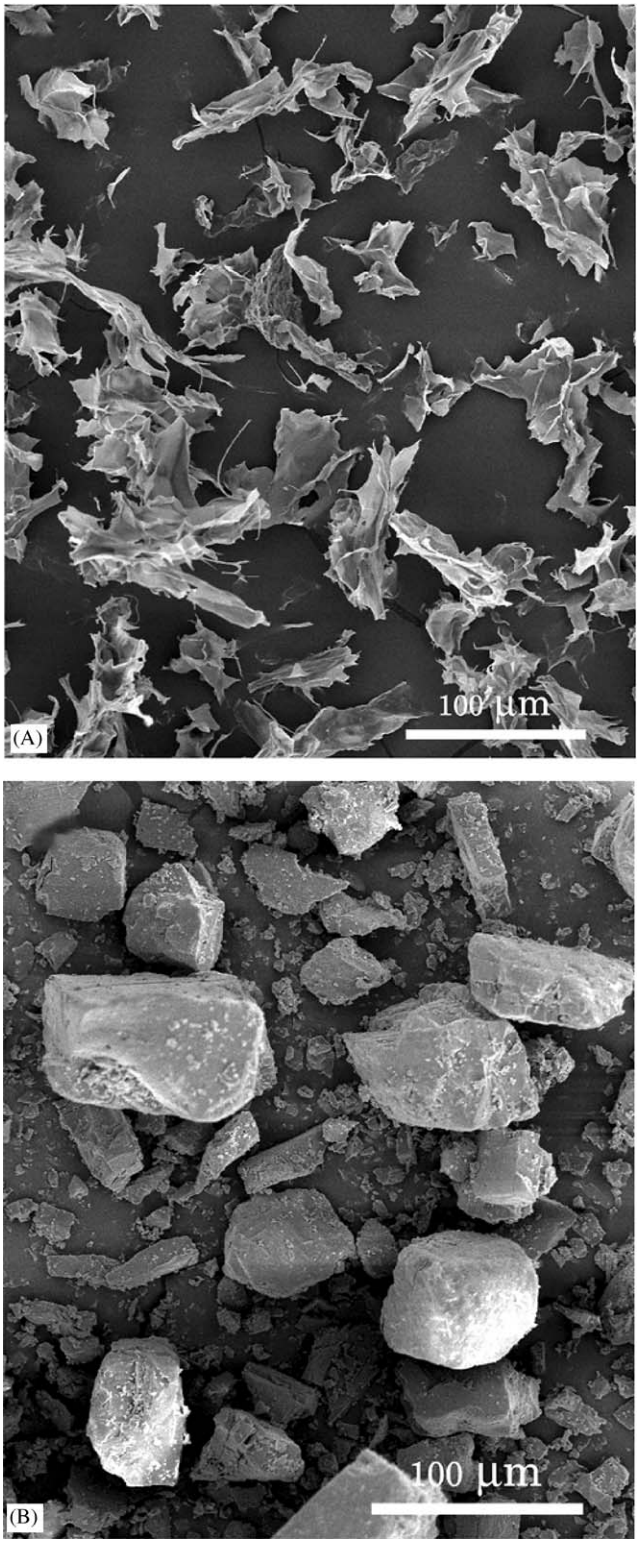

Fig. 1. SEM photographs of (A) pectin particles and (B) NaCl–$CaCl_2$ mixtures (bar = 100 µm).

3.2. Preparation and microscopy of pectin/p(LGA) matrices

The technique described here to prepare pectin/p(LGA) composite matrices stems from the classical procedure termed "solvent-casting and salt-leaching" [23]. In this procedure, salt particles function passively as pore-creating reagents. By the present method, salts play the additional role of a cross-linker, to bridge the highly de-esterified pectin particles. The two processes, salt-leaching and polymer cross-linking, were combined into one step. When a dry, solid matrix of polymer/salt was immersed in water, sodium chloride and calcium chloride dissolved quickly and diffused toward the surrounding liquid phase. Simultaneously, pectin molecules hydrated and swelled slowly as determined by its viscoelastic nature. Normally, the swelled pectin molecules would tend to dissolve and diffuse into the water phase, but they were stopped by the cross-linking with calcium ions to form pectin-calcium hydrogels. The insolubilization of pectin was confirmed by analyzing it in the final matrix (Table 2).

The organization and microstructure of p(LGA) and pectin/p(LGA) matrices are illustrated in Fig. 2. Both matrices were porous and had a sponge-like morphology. Pores were evenly distributed into all areas of the matrices, and they were interconnected. The pores in p(LGA) matrices had sizes and shapes that matched those of the original salt crystals, as evidenced by SEM (Figs. 1B and 2A). Pores and channels in p(LGA) matrices were lined by fibrils or flakes of p(LGA) polymers, indicating the deposition of p(LGA) polymers in the gaps or crevices among salt particles as the solvent evaporated. In this case, the microstructure of the matrices only depends on the weight ratio of salts/polymers and on the particle size of the salt [23].

Topographical, SEM images also revealed that the pores of the composite pectin/p(LGA) matrices were often smaller than the p(LGA) alone, and they were always lined or bordered by smooth, leaf-like surfaces (Fig. 2B). These surfaces resembled the appearance of the image texture of isolated pectin particles used to make the composite (Fig. 1A). These images suggest that the p(LGA) forms a parenchymal matrix, binding the pectin particles which line the pores, together in the composite. Nevertheless, it is difficult to localize the p(LGA) based on image features alone.

Pectin particles were covalently tagged with fluoresceinamine in order to locate areas of p(LGA) indirectly in the composite matrix. Confocal fluorescence and confocal reflection microscopy of the surfaces of composite matrices were used to resolve microscopically the integrated organization of the two components in correlated images. Reflection images (Fig. 3A) in stereo projection reveal the composite structure. Notice

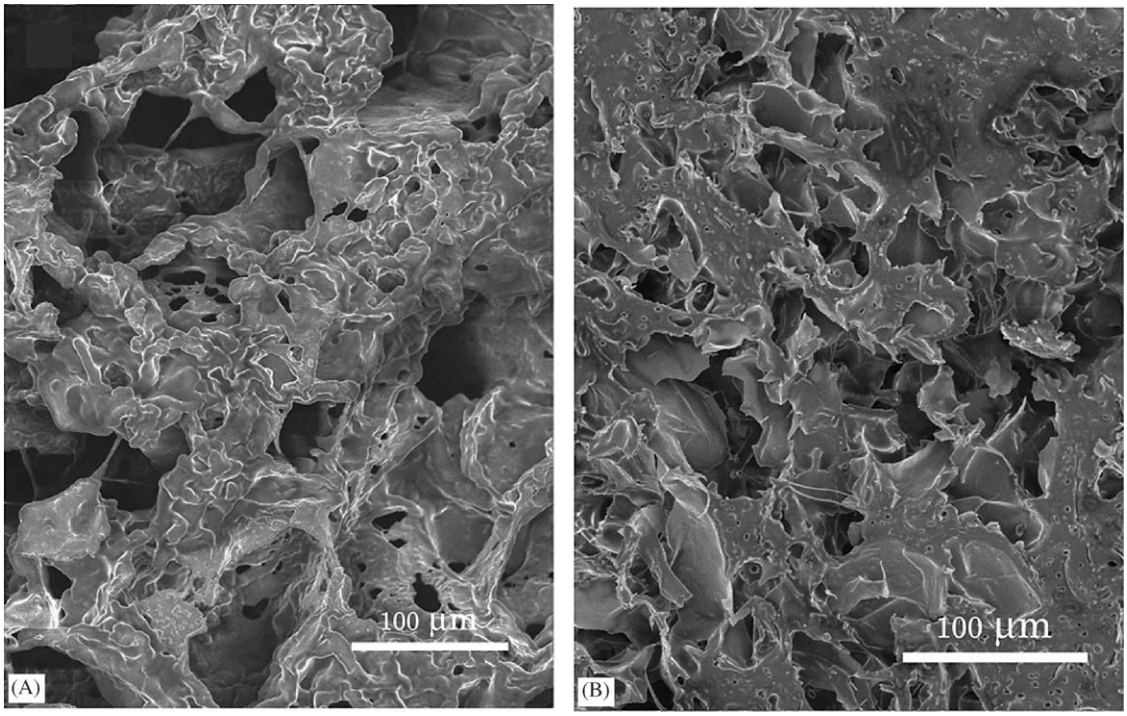

Fig. 2. (A) SEM images of p(LGA) matrix showing a continuous network of salt cavities with the size of 50–200 μm (bar = 100 μm). (B) Pectin/p(LGA) composite matrix showing the leaf- or sheet-like pectin structures which stretched over all space (bar = 100 μm).

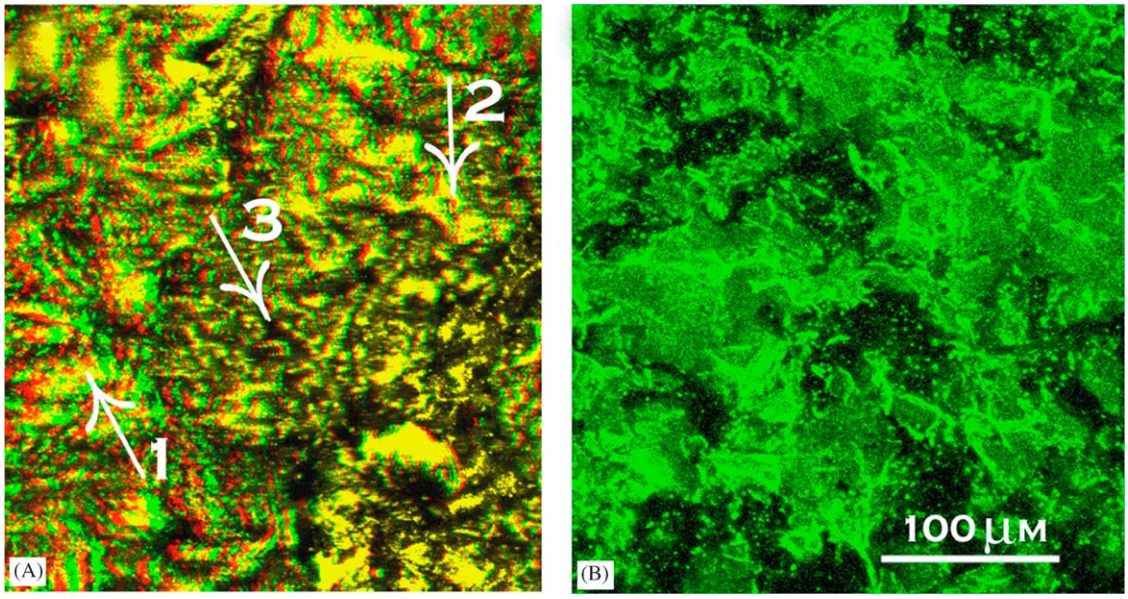

Fig. 3. (A) Averaged confocal reflection images in stereo-projection of pectin/p(LGA) indicating pectin domains constructed with irregular flat sheets with mid-line ridges or small flat patches (1), p(LGA) domains of fine network of anastomosing fibers (2), and the areas of both (3). (B) Laser confocal micrograph of fluorescently labeled pectin/p(LGA) showing the fluorescence located in pectin areas, not in p(LGA) areas (bar = 100 μm).

that a few flat areas of reflection coincide with areas of green fluorescence or pectin (Fig. 3B). This result indicated that whether or not areas of pectin particles reflected light depended upon their orientation. Other areas of reflection, containing irregular tubes and anastomoses do not fluoresce, indicating that these areas contain p(LGA) (Fig. 3A).

From the above observations, it appears that the pectin domains not only filled in the pore spaces created by the deposition of p(LGA) polymers in gaps among particles, but also covered or wrapped most of the p(LGA) domains. In general, pectin/p(LGA) matrices present a complex structure of connected porous pectin networks which are reinforced by p(LGA) networks.

3.3. Dynamic mechanical properties of pectin/p(LGA) matrices

DMA is a useful complement to microscopic methods for morphology and microstrucure investigations of polymeric composites. The dynamic mechanical properties of the pectin/p(LGA) composite matrix and p(LGA) matrix were determined by measuring their compressive storage modulus (E'), loss modulus (E''), and loss tangent ($\tan\delta$). Typical compressive curves are compared for each sample in Fig. 4, along with curves for the pectin alone. The p(LGA) matrix exhibited a noticeable drop in storage modulus starting at about -80°C which then plateau by about -40°C. It also had a sharp glass transition at about 50°C, which is consistent with the data obtained from the supplier. Above this temperature, the sample no longer gave any force readings on the instrument. A sharp peak at about -80°C was seen in the loss modulus curve and the loss tangent curve. The overall trends for the pectin/p(LGA) composite curves are similar to those for p(LGA) curves. Nevertheless, significant differences were noted. The pectin/p(LGA) composite showed a much smaller decline in storage modulus over the -80°C to -40°C range, and had a higher value for the storage modulus over the entire temperature range. It too showed the glass transition at about 50°C. However, above this temperature, the sample still maintained several grams of residual force, whereas the p(LGA) alone retained virtually none. The -80°C peak in the loss modulus was also still visible in the composite, although it was much smaller than in the p(LGA) matrix, and seemed to be smaller than what would be expected from compositional differences alone.

The curves for plain pectin matrices were relatively flat and were comparable to DMA curves obtained for neat pectin films undergoing small deformation dynamic stretching motion [24]. Pectin undergoing dynamic small deformation compression had lower values of storage modulus and loss modulus than the other two matrices below 50°C. However, at about 50°C, the curves for pectin/p(LGA) and p(LGA) matrices had dropped to below the value of the pectin matrix.

The loss tangent behavior of pectin/p(LGA) and p(LGA) matrices was similar to that seen with the loss modulus, although the difference in the peak size at -80°C was more striking. The pectin curve showed a broad peak at about 30°C.

Based on these data, it appears that the presence of the pectin raises the mechanical stiffness of the matrix

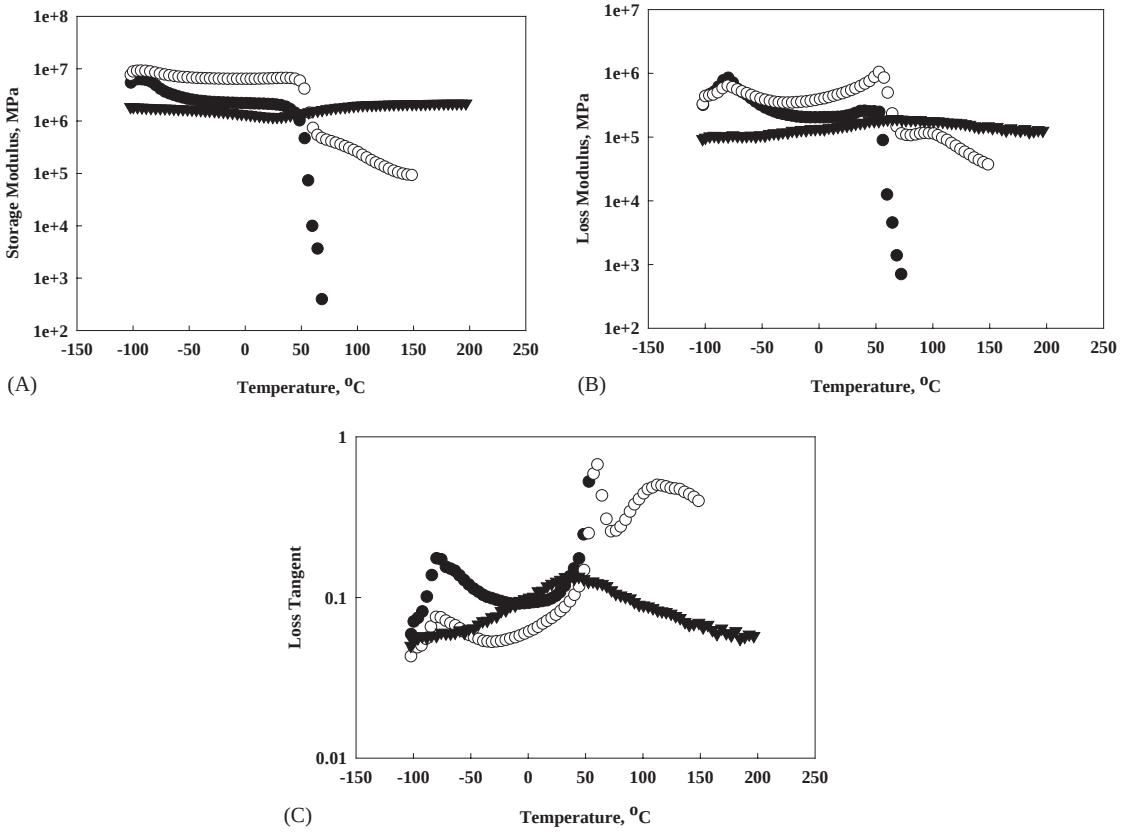

Fig. 4. Typical plots of (A) storage modulus, (B) loss modulus, and (C) loss tangent as a function of temperatures. The pectin/p(LGA) composite matrix (○) has higher storage modulus and loss modulus values than p(LGA) matrix (●) and pectin matrix (▼) in the -80°C to -40°C range. The p(LGA) matrix has a glass transition at about 50°C, above that the p(LGA) give no force reading. In contrast, some residual force still remained with pectin/p(LGA) matrix and pectin matrix. The loss tangent curves of all three types of matrices show a similar trend.

above that of the p(LGA) matrix by itself at temperatures below the glass transition. Above the glass transition, the pectin seems to enable the matrix to maintain some level of physical integrity, although this is at a much lower level than for the matrix at temperature below the glass transition. The incorporation of the pectin network structure seems to be primarily responsible for the increase in the values for E' and for the decrease in the tan δ values for the pectin/p(LGA) composite matrix compared to the p(LGA) matrix. These differences are considered to be the contribution of the well-organized double-network structure of the composite matrices, where the thermoplastic p(LGA) networks were reinforced by the non-thermoplastic pectin networks. The presence of the pectin in the matrix was instrumental in limiting molecular motion of p(LGA) polymers with increasing temperature.

3.4. Characterization of pectin/p(LGA) matrices as carriers of signal molecules

Pectin/p(LGA) matrices were evaluated as carriers of signal molecules by conjugating the matrix with fluoresceinamine (Fig. 3). Green fluorescence was localized in irregular sheets and patches (Fig. 3B). These fluorescent structures are similar to those revealed by SEM for pectin/p(LGA) composite matrices (Fig. 2B), indicating the graft of the fluoresceinamine in pectin areas. Fluorescence was absent from the p(LGA) areas of fibrillar networks in the composite matrices (cf. Figs. 3A and B). This is consistent with the lack of fluorescent emission observed for p(LGA) matrices (data not shown), indicating the inert nature of p(LGA) to the immobilization reaction. The signal molecules of fluoresceinamine were conjugated directly to the sugar rings of pectin via the activation of the hydroxyl groups of the pectins. The hydroxyl groups of carbohydrate molecules are only mildly nucleophilic, approximately equal to water in their relative nucleophilicity. Thus, the activation of pectins was performed in dry acetone to form intermediate reactive derivatives containing good leaving groups for nucleophilic substitution. The reaction of activated hydroxyls with nucleophiles was conducted in PBS (pH 7.8) at room temperature, which resulted in stable covalent bonds between the carbohydrate and the amine-containing molecules [18,19]. Tresyl chloride has been demonstrated to be a useful tool to conjugate various peptides and proteins with synthetic polymers or natural polymers [19,25]. Nevertheless, we observed some loss in matrix integrity in the current experiment, especially when the matrices were treated with dry acetone and during the repeated washing process. It may be due to the differences in swellability between the two networks with medium changes.

Since most signal molecules are environmentally sensitive, the incorporation of signal molecules into biomedical devices is often done under very mild conditions such as in aqueous media, at neutral solution pH, and at 37°C or lower. We evaluated the potential for composite matrices to adsorb signal molecules from aqueous solution by measuring the equilibrium water content and the amount of adsorbed protein. The total water content of matrices was determined by swelling samples of each matrix in PBS and measuring the increase in weight at each incubation time point (Fig. 5A). There was an increase in the water content with the incubation time for both types of matrices. Due to the inclusion of a hydrophilic network, pectin/p(LGA) matrices facilitated water diffusion and uptake into the matrices, as demonstrated by a quick increase in matrix weight at the beginning of incubation. Less time is required to reach equilibrium, and a higher percentage of water adsorbed over the entire time of incubation in comparison with p(LGA) matrices. At steady state, the water content of pectin/p(LGA) composite matrices was about eight-fold of that of p(LGA) matrices (Fig. 6). For protein adsorption, there was a trend similar to water uptake for both pectin/p(LGA) and p(LGA) matrices (Fig. 5B). As in the case of water, the pectin/

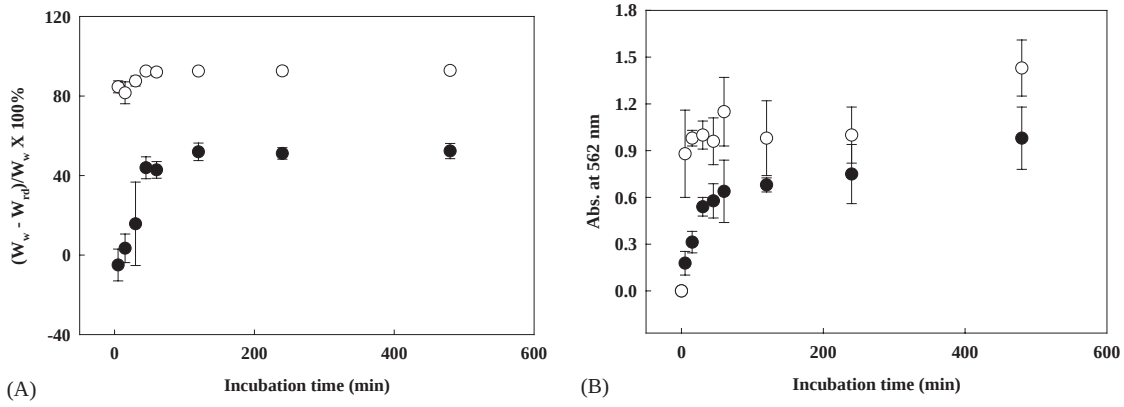

Fig. 5. Time curves of water adsorption (A) and protein adsorption (B) in pectin/p(LGA) matrix (○) and p(LGA) matrix (●). The experiments were conducted at room temperature using PBS as an incubation media. The protein concentration in PBS was 0.1%, w/v.

p(LGA) matrix adsorbed more protein than the p(LGA) matrix (Fig. 6). However, the adsorbed BSA found in pectin/p(LGA) matrices was only 1.5-fold of that detected in p(LGA) matrix (Fig. 6). For p(LGA) matrices, both water and BSA are only able to diffuse to and remain in pore spaces of the matrices. In pectin/p(LGA) matrices, small molecules of water not only diffused and remained in the pore spaces, but also penetrated into the pectin gel domains. Compared to water penetration, only a small fraction of protein BSA was capable of penetrating to the pectin domains.

These results demonstrated the capability of pectin/p(LGA) composite matrices to carry signal molecules either by chemical conjugation or by physical adsorption.

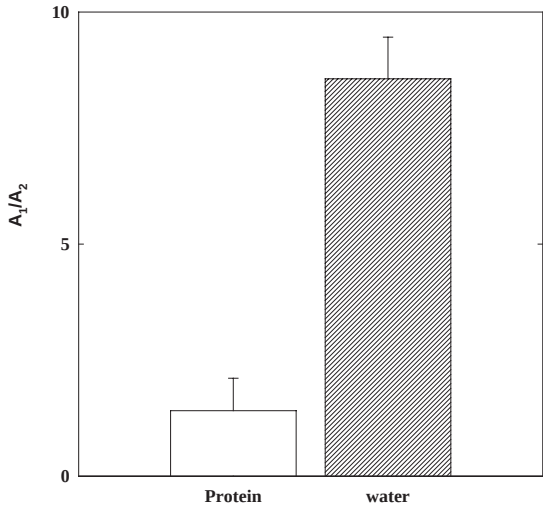

Fig. 6. Comparison of pectin/p(LGA) to p(LGA) matrices in water/protein adsorption. A_1 and A_2: the amount of adsorbates detected at equilibrium in pectin/p(LGA) and p(LGA) matrices, respectively.

3.5. In vitro cell culture

After 1 day of cell seeding, osteoblasts were attached onto pectin/p(LGA) and p(LGA) matrices. There were more cells on pectin/p(LGA) matrices than on p(LGA) matrices. Furthermore, histological analysis revealed that osteoblasts attached to pectin/p(LGA) matrices in multi-layers whereas they attached to p(LGA) matrices in a single layer (Fig. 7). Cells were not only attached on these matrices, but also viable and had the capability to proliferate (Fig. 8). Although the difference in cell number was not statistically significant at the beginning, after 2 weeks culture, cell numbers on the pectin/

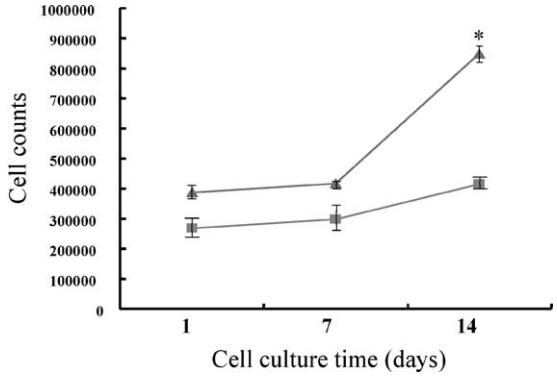

Fig. 8. In vitro osteoblast proliferation on (▲) pectin/p(LGA) and (■) p(LGA) matrices versus cultivation time. One million cells were seeded onto each matrix. $P<0.01$.

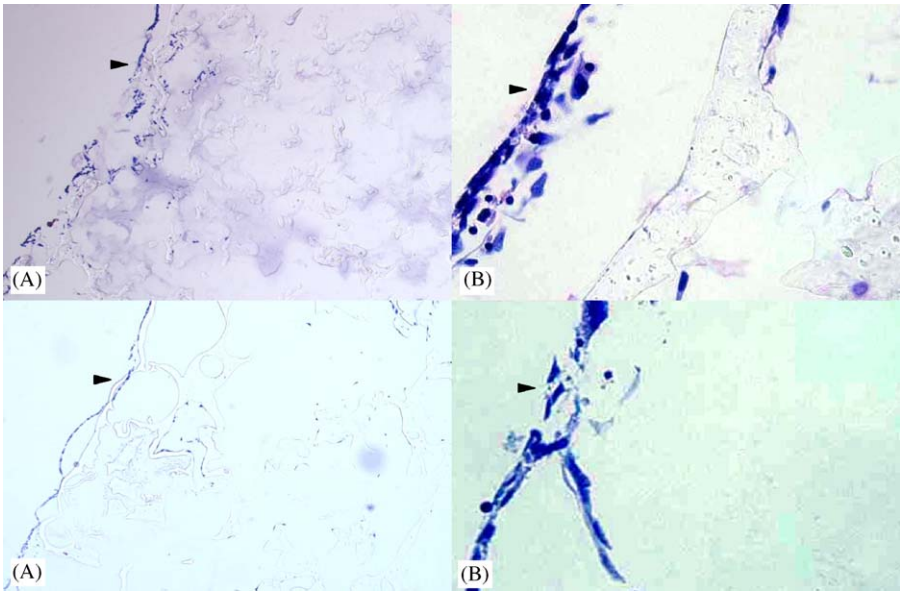

Fig. 7. Osteoblast distribution in pectin/p(LGA) matrices (top panel) and p(LGA) matrices (bottom panel) after 1 day cell seeding. The samples were stained using hematoxylin and eosin. There were more osteoblasts (arrow head) in pectin/p(LGA) matrices than in p(LGA) matrices. Magnification: A (40 ×) and B (200 ×).

p(LGA) matrices were two-fold of that on the p(LGA) matrices (Fig. 8).

4. Conclusions

We present a method to effectively combine synthetic polymers and natural polymers in one matrix. By including dry particles of pectins and calcium chloride in p(LGA)/chloroform solution, composite matrices were created with an interconnected porous morphology. The composite matrices consist of a pectin network reinforced by a p(LGA) network. The composite matrices combine the best features of both polymers. Typically, the mechanical properties of the composite are comparable to p(LGA) whereas their capacity to hold water and accessibility to proteins are comparable to pectin. In addition, the composite matrices provide side chain functional groups for further chemical modifications, which could be used in various biomedical applications. As demonstrated by in vitro cell culture, the composite matrices show promise for tissue engineering applications.

Thus, by selecting a group of synthetic polymers with appropriate pairs of inorganic salts and polysaccharides, many polymeric composite matrices can be created by this simple and environmentally friendly method.

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